Cardiovascular Topics

The role of the endothelium in the reduction of restenosis following balloon angioplasty

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Summary

Objective. **Coronary angioplasty is complicated in one-third of cases by restenosis due to intimal hyperplasia. This is the result of the migration and proliferation of vascular smooth-muscle cells (SMCs) and correlates with the extent of endothelial stripping. To study the effect of rapid re-endothelialisation on preventing intimal hyperplasia, a model of vessel injury is needed which allows for the retention and adhesion of cultured vascular endothelial cells (VECs) in the injured segment.**

Methods. **The abdominal aortas of BD9 rats were injured with an embolectomy catheter and the response to injury assessed on days 1, 3, 7, 14 and 28. Cultured vascular endothelial cells (VECs) were then placed into injured vessels. Introduction of ^{51}Cr-labelled cells was used first-to confirm adhesion, and then unlabelled cells were used to study the effect on intimal hyperplasia.**

Results. **Medial necrosis and complete stripping of the endothelium was seen on days 1 and 3. By day 7 all rats had VECs lining part of the lumen. Intimal hyperplasia was present by day 14. Complete restitution of the endothelium was present at day 28 and medial SMC numbers were also back to normal at this time. Cells producing the intimal hyperplasia were identified as SMCs by staining for smooth-muscle actin and electron microscopy. Re-endothelialisation was confirmed by autoradiography following introduction of ^{51}Cr-labelled VECs. In animals receiving VECs after injury (N = 15), intimal hyperplasia involved a smaller percentage of the lumen circumference compared with controls (N = 15), where only medium was introduced (52.00 ± 6.83% v. 63.47 ± 6.39%; P = 0.03).**

Conclusion. **The response to balloon-induced injury in the rat aorta has been well characterised. This model enabled us to repopulate a damaged vessel with cultured VECs, resulting in a decrease in intimal hyperplasia.**

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Balloon angioplasty is widely used in the management of patients with coronary artery disease, offering a non-surgical therapeutic option to many patients who would otherwise require bypass grafting.[1] However, up to 40% of these procedures fail owing to restenosis at the angioplasty site.[2-5] This is due largely to intimal hyperplasia resulting from the migration and proliferation of vascular smooth-muscle cells

(SMCs).[6-9] Angioplasty causes an increase in lumen cross-sectional area by fracturing the plaque and stretching the disease-free segment of the lumen circumference.[9,10] This procedure invariably results in complete endothelial denudation at the site of the balloon-induced injury.[9-11] Endothelial restitution occurs by regeneration from the ends of the denuded segment.[12-14] This regrowth ceases after 2 weeks in the rabbit and 6 weeks in the rat, leaving permanently denuded segments in vessels where large areas of endothelium have been removed.[14] The extent of endothelial stripping produced correlates well with the degree of intimal hyperplasia which follows. In the rat model it has been shown that intimal hyperplasia only occurs in areas not covered by regenerating endothelium within 7 days.[12] There is also more intimal hyperplasia and more prolonged SMC proliferation in areas which remain devoid of endothelium compared with areas which are eventually re-endothelialised.[13,15]

While it is likely that most models of endothelial denudation cause significant medial damage, the importance of endothelial loss as a pathogenetic factor is emphasised in models of vascular injury where the endothelium is stripped by air desiccation[16] or using a filament loop,[17] causing minimal or no discernible injury to the media but resulting in prominent intimal hyperplasia. We believe that denudation produces stimuli intended to promote endothelial regrowth, which are now able to gain access to deeper layers of the vessel wall, targeting the exposed medial SMCs which then migrate to the intima and proliferate to produce intimal hyperplasia and restenosis. It follows that rapid re-endothelialisation by the introduction of cultured vascular endothelial cells (VECs) into a vessel immediately after injury could prevent intimal hyperplasia and the development of restenosis.

To test this hypothesis models of balloon-induced vascular injury are needed in which the effect of rapid re-endothelialisation on intimal hyperplasia can be studied. Endothelial cell seeding has already been shown to reduce thrombogenicity in endarterectomised baboon aortic segments incorporated into exteriorised arteriovenous shunts[18] and also reduces intimal hyperplasia after endarterectomy in a canine model.[19] The logistical problems presented by larger animal models and the extensive biochemical characterisation of the laboratory rat strongly favour the establishment of a rat model to examine this problem. In this paper we describe a new rat model of restenosis which allows for the delivery and adhesion of cultured VECs to the injured vessel. We also report on the effects of this re-endothelialisation on the reduction of intimal hyperplasia.

Materials and methods

Animals

An inbred strain, the BD9 rat, was used for our model. Male rats weighing 350 ± 50 g were used for all experiments. This study conforms to the guidelines for the care and use of laboratory animals published by the US National Institutes of Health (NIH publication No. 85-23, revised 1985).

Surgery/anaesthesia

The abdominal aorta was exposed by laparotomy under anaesthesia with pentobarbital (Rhône Poulenc, Halfway House, South Africa) 40 mg/kg intraperitoneally.

Balloon-induced injury

Following clamping of the aorta below the renal arteries, a 2F Fogarty embolectomy catheter (Baxter Healthcare Corporation, Santa Ana, USA) was inserted into the abdominal aorta via a small incision just above the iliac bifurcation. Vessel injury was induced by two passages of the inflated balloon. After closure of the arteriotomy, flow was re-established and the abdominal wound sutured.

Monitoring the injury and tissue response

Groups of rats were sacrificed on days 1, 3, 7, 14 and 28 after injury. Each group consisted of 4 rats plus 1 control in which the aorta was clamped without ballooning being performed. Animals were killed while under pentobarbital anaesthesia by perfusion with phosphate-buffered saline (PBS) via a large cannula placed in the left ventricle. The left atrium was cut to allow for drainage. This was followed by perfusion of the aorta with 2.5% glutaraldehyde. The infra-renal abdominal aorta was recovered and further fixed by overnight immersion in the same fixative. Arterial cross-sections cut from paraffin blocks containing the middle third of these vessels were processed for standard haematoxylin and eosin staining as well as electron microscopy.

To facilitate the identification of the different cell types, several sections of the paraffin-embedded tissue were stained with antibodies to smooth-muscle actin. The sections were dewaxed with xylene, hydrated with ethanol and washed in distilled water. Endogenous peroxidase was blocked with 3% hydrogen peroxide for 5 minutes. Sections were then washed in phosphate-buffered saline (PBS), pH 7.6, at room temperature. The primary antibody, against smooth-muscle actin (clone 1A4, Cat. No. M851, Dako, Glostrup, Denmark), was applied at a dilution of 1:200 and incubated for 30 minutes. After 3 washes biotinylated rabbit anti-mouse link antibody (Dako, Denmark, Cat. No. A354) was applied at a dilution of 1:250 for 30 minutes. After washing, sections were incubated with horseradish peroxidase conjugated streptavidin-biotin complex (Dako, Denmark, Cat. No. P397) for 30 minutes. Sections were washed as before and positive labelling demonstrated using 0.05% 3.3'-diaminobenzidine tetrachloride (DAB) in PBS, pH 7.6 (160 μl of 3% H_2O_2 was added to 50 ml of the DAB solution just before use) for 3 - 5 minutes. Sections were then washed in running tap-water, counterstained with Mayer's haematoxylin, dehydrated, cleared and mounted in DPX. While the Dako cross-reactivity chart shows conflicting data on cross-reactivity with rat tissue, we found consistent positive staining of medial SMCs. Negative controls, where the primary antibody was replaced with normal serum, were also included.

Morphometric analysis was then done to assess the extent of denudation, spontaneous re-endothelialisation and

intimal proliferation. Complete arterial cross-sections were photographed at a magnification of × 25 and 8 × 10 inch black-and-white enlargements were obtained. The degree of intimal proliferation was assessed by the following three measurements: the circumference affected by intimal hyperplasia was measured and reflected as a percentage of total lumen circumference; the maximal intimal thickness was reflected as a percentage of total wall thickness at that point; and intimal area was reflected as a percentage of total vessel wall area. This last ratio was obtained by carefully cutting out and weighing the areas representing the intima and media and calculating the intimal area as being equal to the weight of the intima × 100 divided by the weight of the combined intima and media. This method is based on that used by Haudenschild and Schwartz.[12]

Cell culture

Microvascular endothelial cells were prepared from the ventricles of young BD9 rats (mass 150 - 200 g) using Langendorff perfusion with a 0.1% collagenase solution (Boehringer Mannheim GmbH, Mannheim, Germany) as described by Piper *et al.*[20] Cells were grown to confluency in complete medium (medium 199 with 10% fetal calf serum (Highveld Biological (Pty) Ltd, Kelvin, South Africa), penicillin (Beecham Pharmaceuticals (Pty) Ltd) and streptomycin (Sigma Chemical Co., St Louis, USA)) at 37°C and 5% CO_2. Verification that the cells in culture were indeed VECs included the use of electron microscopy as well as the presence of positive staining for von Willebrand factor. After one to three passages, cells were recovered by trypsinisation (0.05% trypsin-versene mixture, Bio Whittaker, USA) and resuspended in complete medium. Cell counts were performed using a haemocytometer. Cells were then pelleted at room temperature by centrifugation for 10 minutes at 1 000 rpm and resuspended in PBS at a concentration of 10^6/ml. Calcium chloride was added to achieve a Ca^{++} concentration of 1.2 mmol/l and the suspension was kept at 37°C until used. In most cases these suspensions of VECs were used within 2 hours of preparation and in all cases within 3 hours.

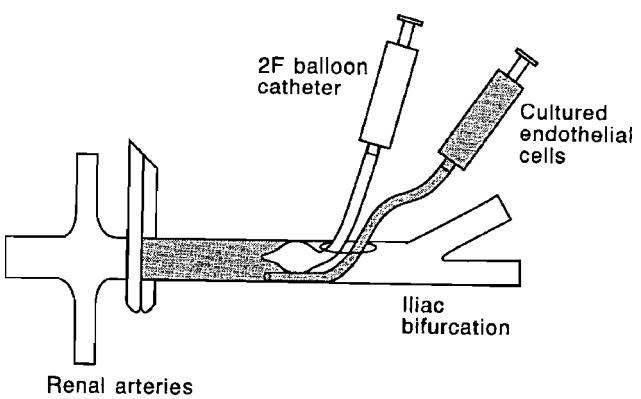

Fig. 1. Schematic diagram of the rat aorta model of vascular injury. The area of the abdominal aorta between the clamp near the renal artery and the incision near the iliac bifurcation has been injured by two passages of an inflated balloon catheter and is devoid of endothelium. Cultured VECs are introduced into the injured segment which is isolated by inflating the balloon just above the incision. After time is allowed for adhesion the incision is closed and flow re-established.

Re-introduction of endothelial cells

Following balloon injury the Fogarty catheter was placed so as to position the balloon just above the arteriotomy. Resuspended cells were drawn up into a 1 ml syringe then introduced into the aorta through a 22F cannula which entered the injured segment via the arteriotomy. This arterial segment was isolated by the clamp below the renal arteries and the inflated balloon above the arteriotomy near the iliac bifurcation (Fig. 1). A volume sufficient to cause slight distension of the isolated segment was used. Fifteen minutes was allowed for adhesion of the cells before the arteriotomy was closed and flow re-established.

Monitoring the success of re-endothelialisation

Cell adhesion was monitored following introduction of ^{51}Cr-labelled VECs. Cultured VECs were detached by trypsinisation, resuspended in 0.5 ml complete medium, then labelled by incubation with ^{51}Cr (Amersham, Bucks, UK) at 37°C for 30 minutes using 25 μCi/10^6 cells. The suspension was pelleted by centrifugation for 10 minutes at 1 000 rpm and after two washes with PBS the cells were resuspended at 10^6/ml in PBS/1.2 mmol/l Ca^{++}. An aliquot of this suspension was pelleted and the cell-free supernatant containing radiolabel used in one of our control groups (group A). Animals were sacrificed 1 hour after the surgery. The infra-renal abdominal aortas were recovered and opened longitudinally. Following mounting on blotting paper and drying in a slab gel dryer (model SE450, Hoefer Scientific Instruments, USA) the aortas were subjected to *en face* autoradiography using Kodak X-Omat film (Eastman Kodak, New York, USA). This was exposed for 14 days at –70°C. Three groups of animals were evaluated — a control group where injury was followed by introduction of cell-free medium containing free radiolabel only (group A), an experimental group where injury was followed by the introduction of ^{51}Cr-labelled cells (group B), and a second control group where labelled cells were introduced but injury omitted (group C). In each case the medium or cell suspension was incubated in the isolated aortic segment for 15 minutes.

Monitoring the effects of re-endothelialisation

Unlabelled VECs were used for this part of the study. Rats in which cultured VECs were introduced after balloon injury (treatment group, N = 15) were compared with animals in which only cell-free medium was introduced after injury (control group, N = 15). Animals were sacrificed 2 weeks after injury and the degree of intimal proliferation assessed histologically as described above.

Statistical analysis

Results were not assumed to be normally distributed and are presented as mean values ± standard error of the mean. Non-parametric analysis was used to compare measurements in the treatment and control groups (Wilcoxon signed rank test).

Results

Response to injury

The morphology of the injured vessels was remarkably similar within each group. All the control animals demonstrated normal morphology with a monolayer of endothelial cells forming the intima and a media which was well populated with SMCs (Fig. 2). Complete stripping of the endothelium and extensive medial necrosis was seen in all rats sacrificed on days 1 and 3 (Fig. 3). The media was rendered virtually acellular with very few nuclei still visible. In all animals the luminal surface of the internal elastic lamina was covered in places by a single layer of platelets. Medial haemorrhage and formation of intraluminal thrombus was occasionally present. By day 7 all animals had vascular endothelial cells (VECs) lining part, but not the entire circumference, of the lumen (Fig. 4). Intimal hyperplasia was seen in 1 of the 4 rats sacrificed on day 7. Intimal hyperplasia was present by day 14 in all rats (Fig. 5). At this time regeneration of endothelium was far advanced in all animals, lining most, but not all, of the vessel circumference. Complete restitution of the endothelium was present at day 28 and medial SMC numbers, which had started increasing by day 14, appeared to be back to normal at this time (Fig. 6). Cells producing the intimal hyperplasia were shown to be SMCs on the basis of their appearance on electron microscopy as well as their positive staining for smooth-muscle actin (Fig. 7).

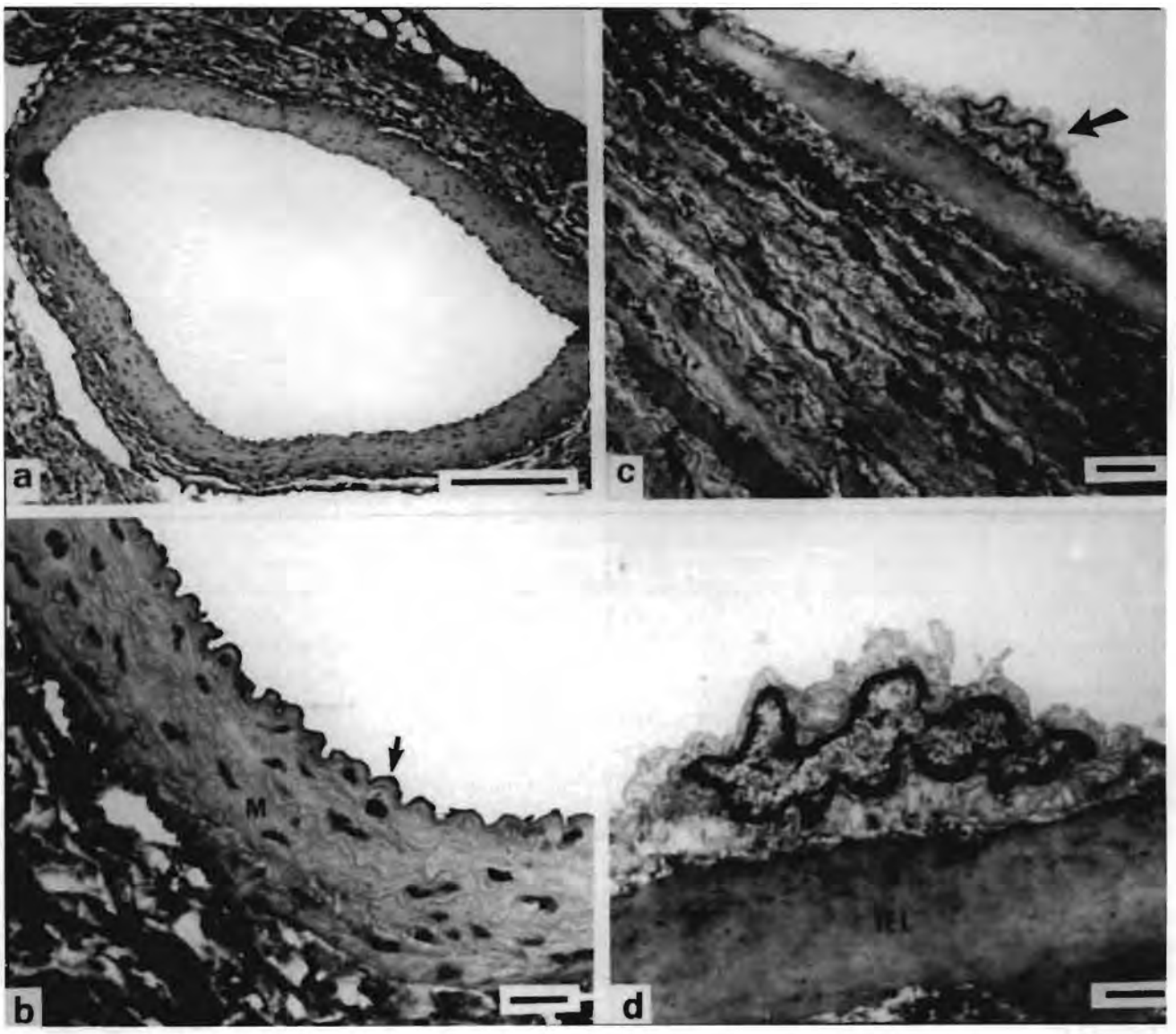

Fig. 2, a - d. Normal aortic morphology is demonstrated in the control animals by light microscopy at a final magnification of × 180 (a, bar 30 μm) and × 1 200 (b, bar 8 μm), and by electron microscopy at a final magnification of × 5 250 (c, bar 2 μm) and × 15 000 (d, bar 0.7 μm). As demonstrated, the intima consists of a single layer of flattened endothelial cells (arrows) lining the internal elastic lamina (IEL), and the media (M) is well populated with SMCs.

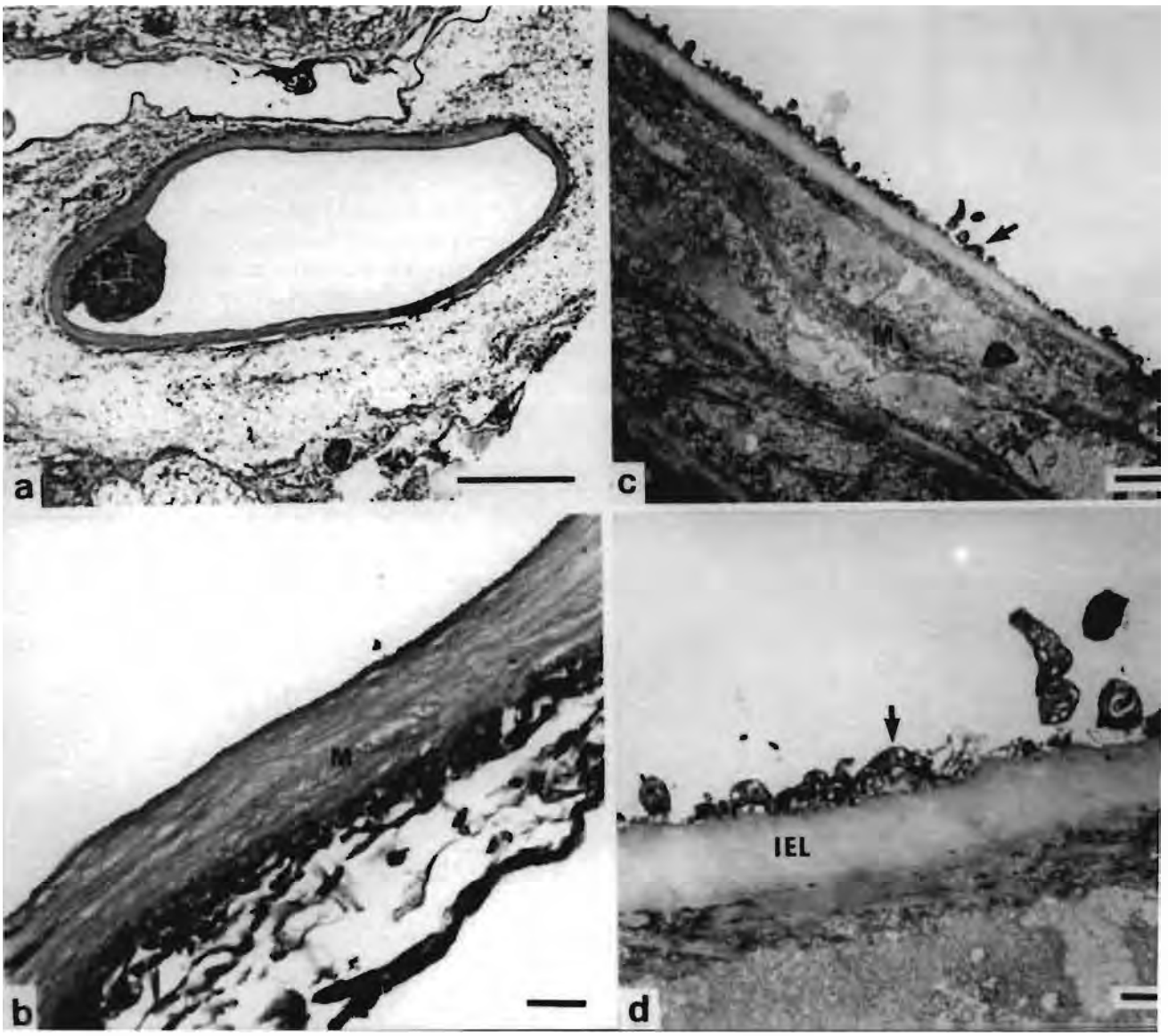

Fig. 3, a - d. The morphology as seen 3 days after injury (identical to that seen on day 1). The light microscopic appearance is demonstrated at a final magnification of × 180 (a, bar 30 μm) and × 1 200 (b, bar 8 μm), while the electron microscopic appearance is shown at a final magnification of × 2 250 (c, bar 4 μm) and × 9 000 (d, bar 1 μm). There has been complete endothelial stripping with only a monolayer of platelets (arrows) lining the internal elastic lamina (IEL). The media (M) is severely damaged with no SMC nuclei visible. A small mural thrombus can be seen (a).

Adhesion of cultured VECs

The presence of positive staining for von Willebrand factor and the electron microscopic appearance of the cultured cells used confirmed that they were indeed endothelial cells. Two sets of experiments using autoradiography confirmed cell adhesion after their introduction into injured vessels with a strongly positive radiograph of injured aortas exposed to ^{51}Cr-labelled cells, minimal uptake of label when injured aorta was exposed to cell-free supernatant containing free radiolabel, and virtually no uptake when non-injured aortas were exposed to labelled cells (Fig. 8).

Effect of re-endothelialisation on intimal hyperplasia

For this part of the study unlabelled cells were used and all animals were sacrificed 14 days after injury, a time when intimal hyperplasia is well established. In the group receiving cultured VECs after aortic injury (N = 15), intimal hyperplasia involved a significantly smaller percentage of the total lumen circumference compared with the control animals (N = 15), in which only medium was introduced after injury (52.00 ± 6.83% v. 63.47 ± 6.39%; P = 0.03). Maximal intimal thickness also formed a smaller proportion of total wall thickness (50.00 ± 5.92% v. 52.56 ± 5.85%; P = 0.71) and intimal area comprised a smaller percentage of total vessel area (22.98 ± 3.42% v. 31.00 ± 5.77%; P = 0.71) in the treatment group (Fig. 9), although these differences were not statistically significant.

Discussion

Intimal hyperplasia or myo-intimal proliferation appears to be the manner in which the vessel wall responds to a num-

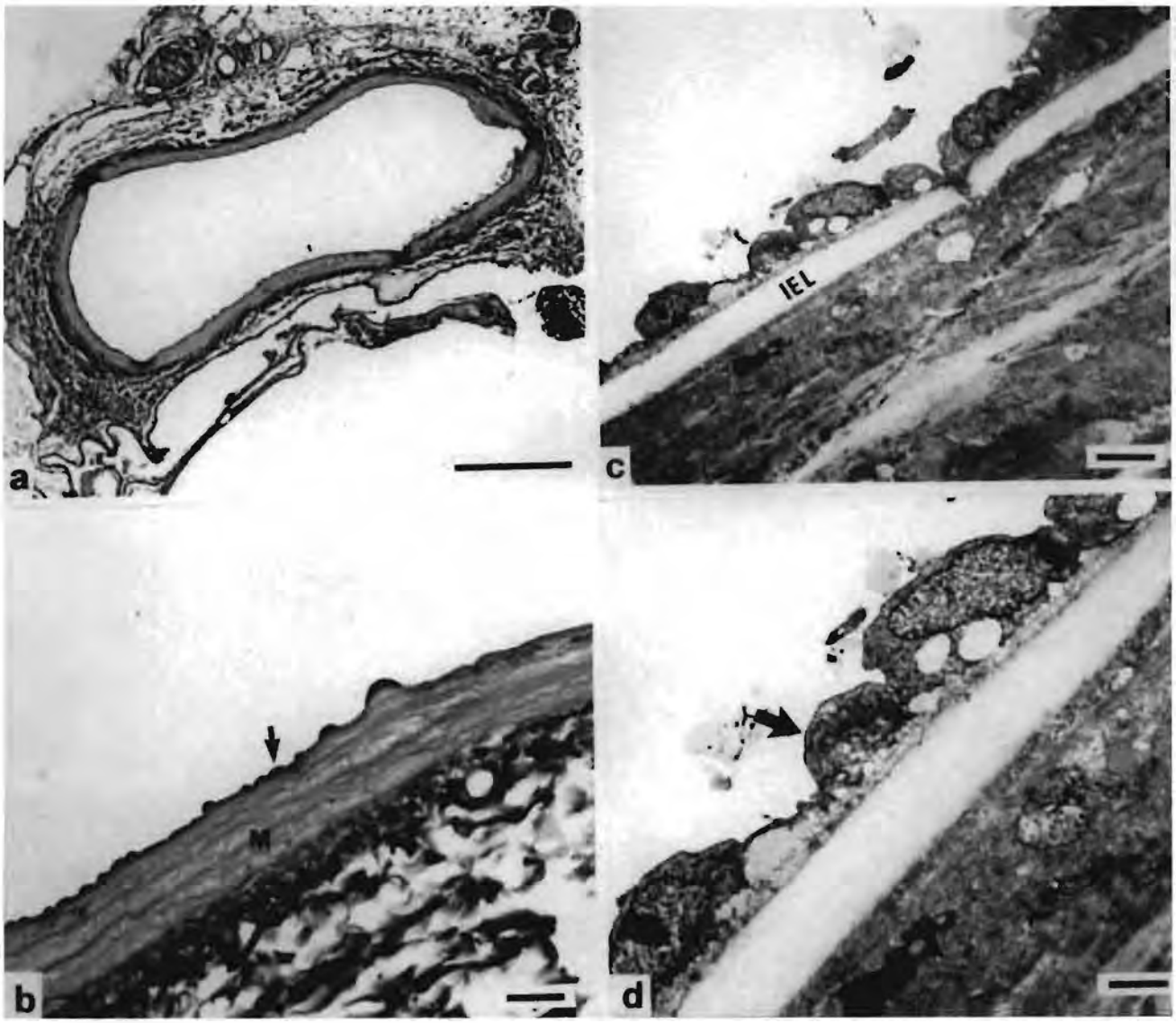

Fig. 4, a - d. Day 7 after injury. The light microscopy is shown at a final magnification of × 180 (a, bar 30 um) and × 1 200 (b, bar 8 µm), while electron micrographs are shown at a final magnification of × 2 250 (c, bar 4 µm) and × 5 250 (d, bar 2 µm). Endothelial regrowth has begun and lines part of the lumen (arrows). The media (M) remains acellular at this point.

ber of different forms of injury. Physical, chemical or biological trauma all produce similar results.[21] Balloon angioplasty of atherosclerotic coronary artery lesions is also followed by intimal hyperplasia and restenosis in about one-third of cases, thereby limiting the clinical efficacy of this intervention.[2-5]

The pathogenesis of restenosis is clearly multifactorial.[22] Injury results in the release of a number of growth factors from damaged medial SMCs, adjacent VECs and platelets adherent to the damaged intimal surface.[23,24] This growth factor 'cascade' includes platelet-derived growth factor,[23-25] basic fibroblast growth factor,[26-30] transforming growth factor β,[25-31] angiotensin II,[32] endothelin[33-35] and others, and causes endothelial regrowth, platelet aggregation, SMC migration, SMC proliferation and deposition of connective tissue matrix. The loss of endothelium results in reduced levels of 'protective' factors such as heparin,[36-39] nitric oxide[40-42] and prostacyclin.[43-45] The net effect is thrombus formation at the site of injury, migration of medial SMCs into the intima and the adherent thrombus, and then intimal proliferation of SMCs to cause restenosis.[9,15,46,47] Much attention is now also being focused on the chronic vasoconstriction or vascular remodelling which occurs after arterial injury and also contributes significantly to reductions in luminal diameter.[48-53]

Given this complex pathogenesis, it is not surprising that no single intervention has consistently reduced restenosis rates in human controlled trials. In particular, many pharmacological agents directed against specific growth factors have been successful in various animal models but proved ineffective when tested on human subjects.[22,54,55] We have investigated the hypothesis that rapid restitution of the endothelial lining of the injured vessel by seeding with cultured VECs immediately after injury may inhibit this growth factor cascade and reduce restenosis. Rapid restitution of the endothelium will shield SMCs from serum growth factors, and restore to the vessel wall those factors that favour endothelial proliferation, anticoagulation and vasodilatation and inhibit SMC migration and proliferation.

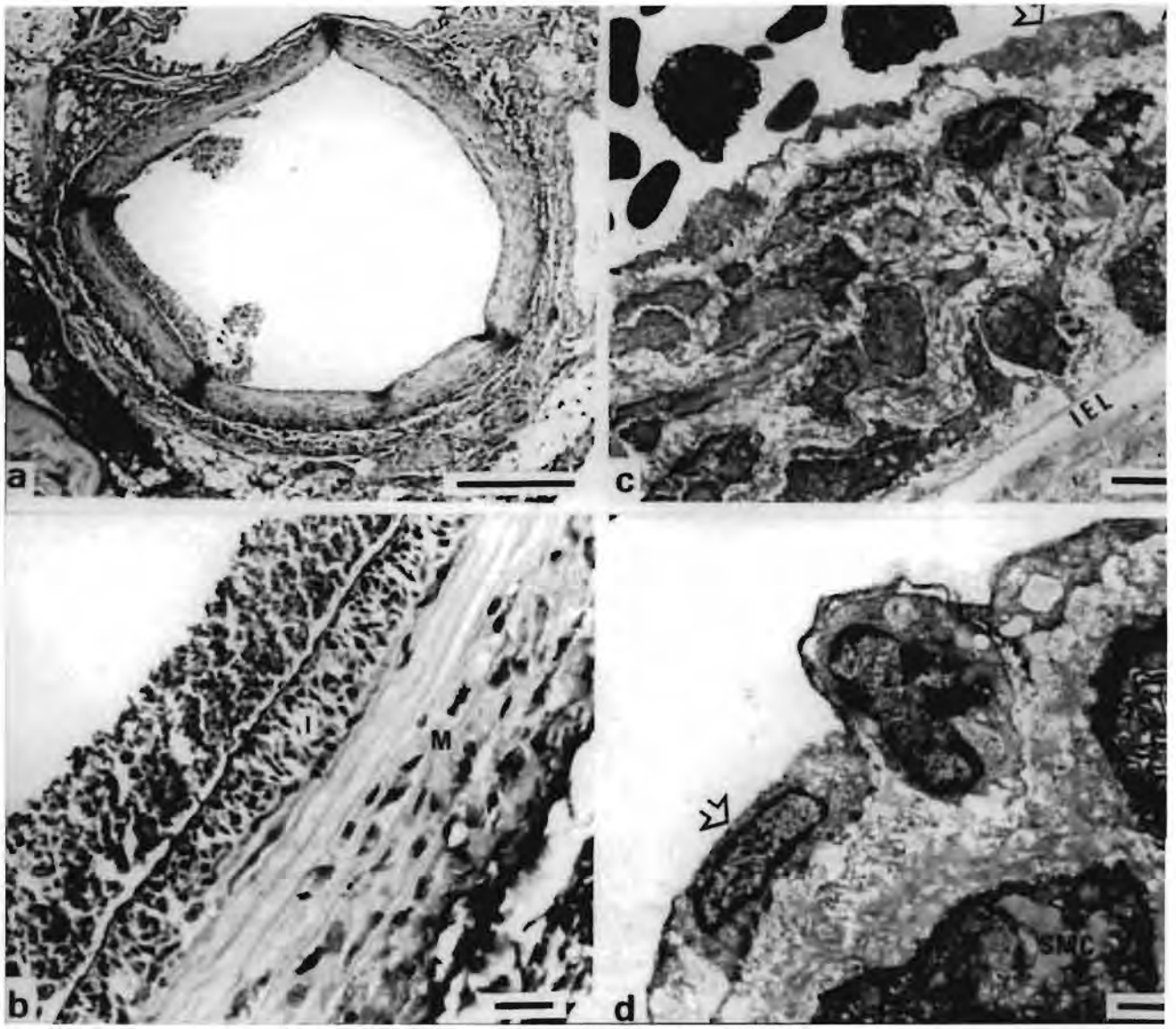

Fig. 5, a - d. Day 14 after injury. The light microscopy is shown at a final magnification of × 180 (a, bar 30 um) and × 1 200 (b, bar 8 µm), while electron micrographs are shown at a final magnification of × 3 750 (c, bar 2.7 um) and × 7 500 (d, bar 1.3 µm). Most of the lumen circumference is now lined with endothelial cells (arrows). Intimal hyperplasia (I) is present in all animals with SMCs occupying the area between the internal elastic lamina (IEL) and the luminal lining of endothelial cells. Some SMCs are also starting to repopulate the media (M).

Several studies have provided support for this hypothesis. Endothelial seeding of prosthetic vascular grafts was introduced in 1978 and has since been shown to increase patency and decrease thrombogenicity in both animal and human studies.[56] Seeding of damaged native vascular surfaces has been performed using endarterectomised arterial segments[18,57] following endarterectomy in a canine model[19,58] and following angioplasty in the rabbit model.[59,60] In the Yucatan minipig, Nabel *et al.*[61] described the use of a double-balloon catheter to isolate an arterial segment injured by angioplasty so as to permit the delivery and attachment of genetically modified vascular endothelial cells.[1] The studies using seeding of endarterectomised arterial segments with high concentrations of VECs (sodding) demonstrated that acutely attached VECs markedly reduce platelet deposition and rapidly spread out to produce a confluent monolayer under conditions of high blood flow rates. In the rabbit model Conte *et al.*[60] achieved 40 - 90% coverage of the denuded surface with *in vivo* delivery of genetically modified VECs. In the same model Conte's group then showed that restoration of the endothelial monolayer was accelerated by endothelial cell seeding, but did not find evidence of a reduction in intimal thickening.[62] After carotid endarterectomy in the canine model, Bush and co-workers[19,58] found that treatment with cultured autologous vascular endothelial cells accelerated healing of the luminal surface, restored prostacyclin levels in the vessel wall and inhibited the development of intimal hyperplasia. To our knowledge the study by Conte *et al.*[62] is the only one which has reported the effects of treatment with cultured VECs after balloon angioplasty on the development of intimal hyperplasia, and none has examined this intervention in a rat model.

In the established rat models of restenosis (balloon injury to carotid or thoracic aorta), isolation of the injured arterial segment to allow for the introduction and retention of cultured vascular endothelial cells is not practical. The models

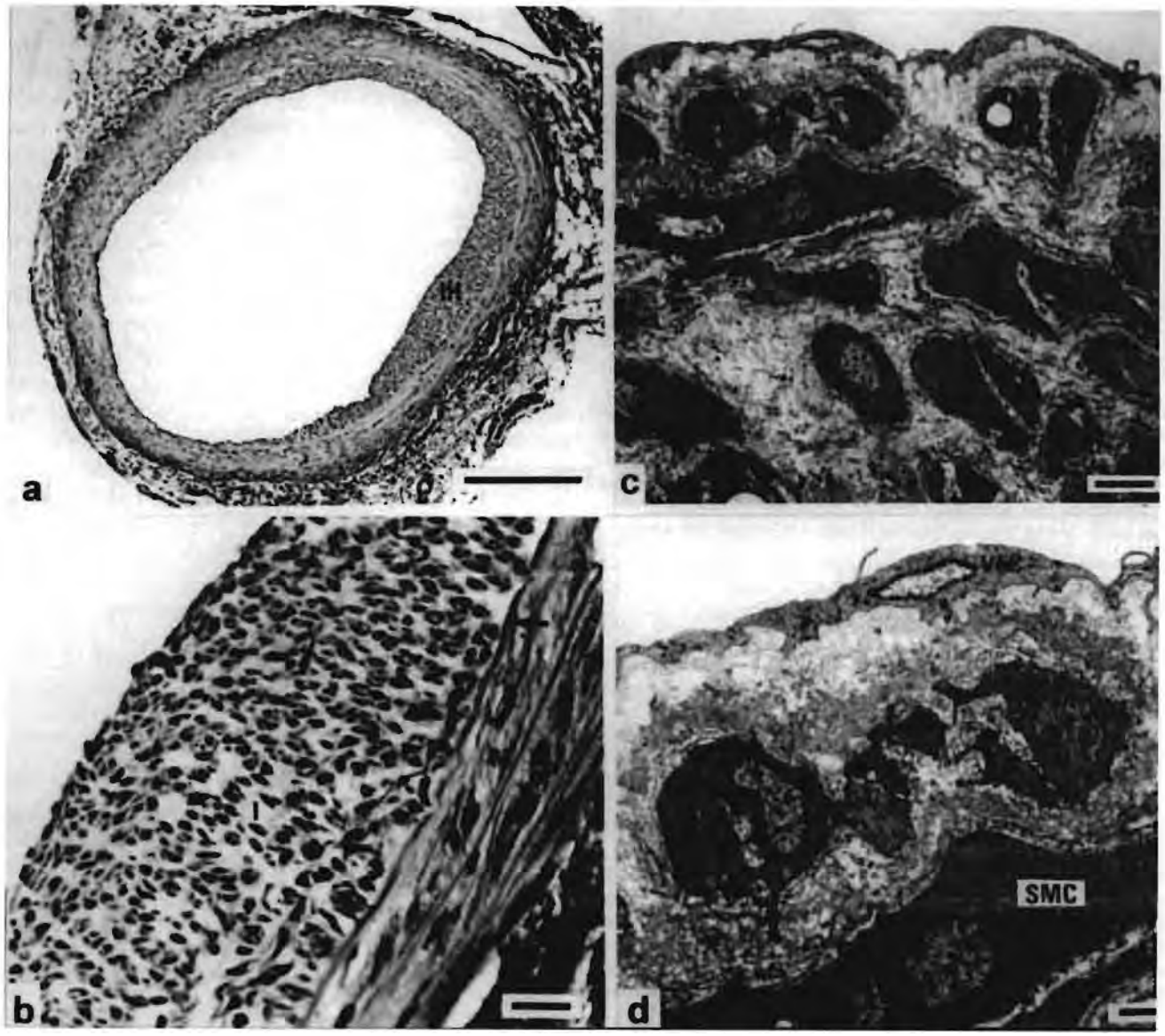

Fig. 6, a - d. Day 28 after injury. The light microscopy is shown at a final magnification of × 180 (a, bar 30 µm) and × 1 200 (b, bar 8 um), while electron micrographs are shown at a final magnification of × 2 250 (c, bar 4 µm) and × 5 250 (d, bar 2 µm). In comparison with the response at 14 days, the intimal hyperplasia (IH) is more pronounced and is seen to be distributed in an eccentric pattern (a). The increase in cellularity of the intima (I), separated from the media (M) by the internal elastic lamina (b, arrow) is clearly seen. This is due to the presence of SMCs (d). Note that the media too shows an increase in the number of SMCs present.

are based on that of Baumgartner,[63] who injured the rabbit abdominal aorta and jugular vein with a balloon catheter by gaining access via a branch or tributary which was ligated after the injury was performed and the catheter withdrawn. In these rat models the balloon catheter is introduced via an incision in the common carotid or external carotid artery which has been ligated distally. After the catheter is passed proximally and the common carotid or thoracic aorta injured, it is withdrawn and the vessel through which it was introduced tied off proximal to the incision.[13,14,21,64] The seeding of injured vessels with VECs has therefore been studied in larger animals where the injured vessel could be isolated relatively easily. However, the logistical problems presented by larger animals and the fact that most of the work on restenosis has been done in the laboratory rat strongly favoured the establishment of a rat model to examine this problem.

The response to balloon-induced injury in the rat aorta has been well characterised in this study. Our findings are similar to those obtained by Clowes *et al.*[13] with the rat carotid artery model and Schwartz *et al.*[21] with the rat thoracic aorta model. Endothelial regrowth was first seen on day 7 and was complete by day 28. The study by Schwartz *et al.* showed that although the endothelium appeared to have regrown by 4 weeks at the light microscopic level, electron microscopy demonstrated that the endothelium does not become continuous until 2 months after injury. We found that intimal hyperplasia had started in some rats by day 7, was present in all animals by day 14 and had become more prominent by day 28. It has previously been shown that the continued intimal thickening after 2 weeks is due to the synthesis and accumulation of connective tissue and not to an increase in SMC numbers.[13]

This model has enabled us to repopulate a damaged ves-

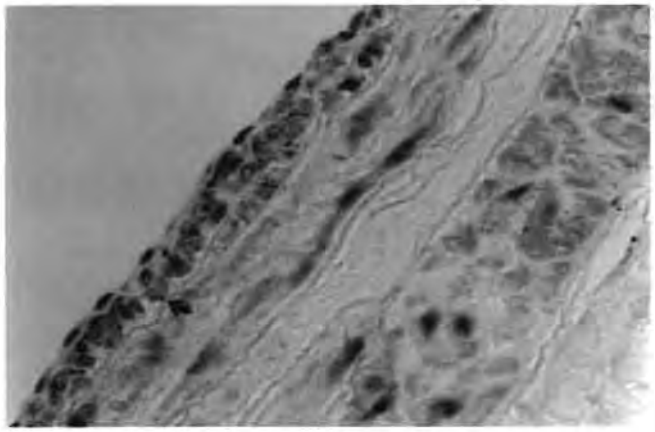

Fig. 7. Staining with antibodies to smooth-muscle actin using the streptavidin method confirms that the cells producing the intimal hyperplasia are of SMC origin. Note the prominent staining of the cells in the intima and media but virtually absent staining of the endothelial cells lining the lumen (final magnification × 1 600).

Fig. 8. Adhesion of ^{51}Cr-labelled VECs to the injured vessel. Two representative examples of the results obtained are demonstrated. Strong positivity is observed when an injured aorta is incubated with labelled cells (B). When labelled cells are introduced without prior injury (C) or when cell-free ^{51}Cr is introduced into an injured vessel (A) there is minimal uptake of radiolabel.

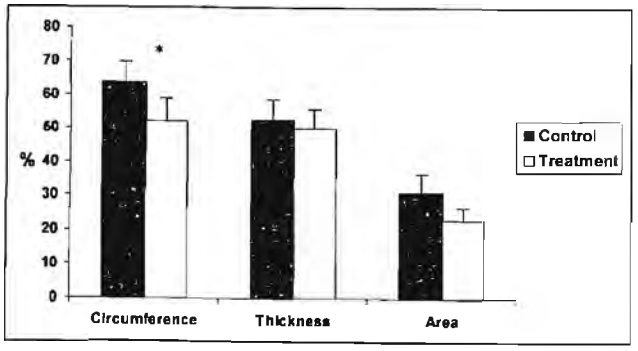

Fig. 9. Bar graph showing the effect of re-endothelialisation on intimal hyperplasia. In animals treated with VECs there is less intimal hyperplasia after balloon injury. Less of the circumference of the vessel is involved by intimal hyperplasia (52.00 ± 6.83% v. 63.47 ± 6.39%; $P = 0.03$). Maximal intimal thickness also forms a smaller proportion of total wall thickness (50.00 ± 5.92% v. 52.56 ± 5.85%; $P = 0.71$) in the treatment group and intimal area comprises a smaller percentage of total vessel area (22.98 ± 3.42% v. 31.00 ± 5.77%; $P = 0.71$).

sel with cultured VECs and study the effects of this intervention on intimal hyperplasia. Re-endothelialised rats developed less intimal hyperplasia after balloon-induced vessel injury (Fig. 9). The degree of intimal hyperplasia as judged by the percentage of vessel circumference involved was reduced by 18% ($P = 0.03$), a statistically significant effect. The reduction in hyperplasia as judged by maximal intimal thickness (5%) and intimal area (2%) did not reach statistical significance, although the trend in both cases was in favour of a beneficial effect of re-endothelialisation.

Optimising the seeding procedure by seeding at high density, improving cell adhesion or accelerating the proliferation of attached endothelial cells by the addition of growth factors may well produce a statistically significant reduction in intimal hyperplasia and will be incorporated into future studies. Although these data cannot as yet be extrapolated to the clinical situation, this study provides sufficient evidence to support further studies into the role of the endothelium in the prevention of restenosis. While there is a need to look at ways of optimising the seeding procedure, at the same time the mechanisms whereby the endothelial cell protects against intimal hyperplasia deserve further scrutiny.

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